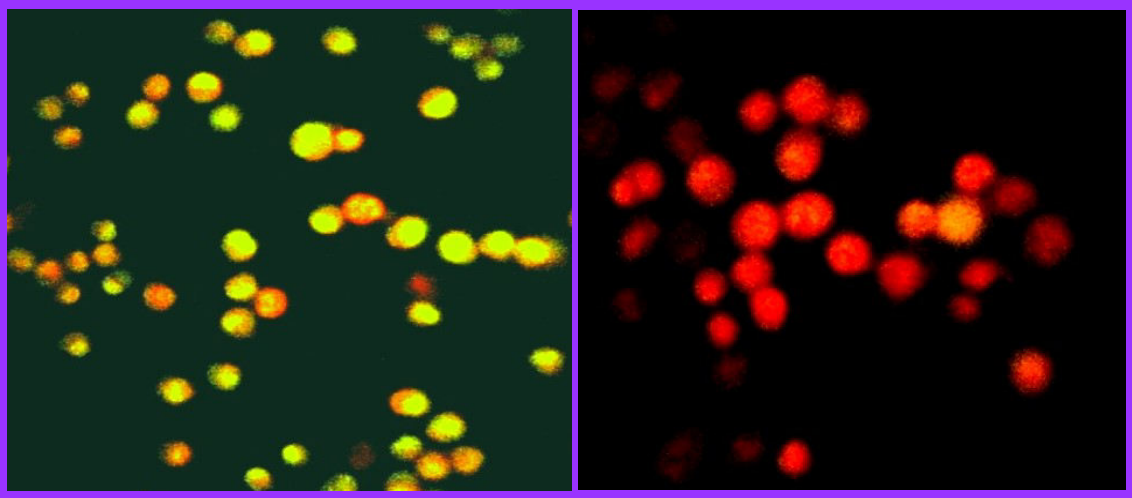

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Anti-cancer potential of banana flower extract: An *in vitro* study

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Abstract

Banana (*Musa paradisiaca)* flower is rich in phytochemicals (vitamins, flavornoids, proteins) and has anti-oxidant properties. The anti-cancer activity of banana flower extract has been evaluated on the cervical cancer cell line HeLa. The anti-proliferative effects were evaluated by MTT assay. The extract was further purified by TLC and characterized by LC-MS method. The ethanol extract had significant cytotoxicity to HeLa cells with an IC_{50} of 20 µg/mL. By thin layer chromatography we could isolate three fractions out of which fraction 2 had exhibited maximum anti-proliferative effects with an IC_{50} value of <10 µg/mL. By LC-MS analysis, bioactive fraction was found to have an m/z value of 224.2 indicating it as a novel one.

Introduction

There is a large amount of experimental evidence which suggests that consumption of fruits and vegetables lower the risk of cancer (Chen et al., 2004). Phenolic compounds are one of the most abundant and ubiquitous group of plant metabolites, and are an integral part of the human diet. In addition to their primary potent anti-oxidant activity, this group of compounds display a wide variety of biological functions, which are mainly related to intervention in various stages of cancer development including initiation, progression, promotion, invasion and metastasis (Kampa et al., 2007; Ramos, 2008). *Musa* sp. (Musaceae) also known as banana is a familiar tropical fruit and important source of food in the world. From its native South western Pacific home, the banana plant spread to India by about 600 BC and later on it spread all over the tropical world. It is possibly the world's oldest cultivated crop (Yusoff, 2008). It possesses efficient medicinal values such as stem juice is used in nervous affectations like epilepsy, hysteria and in dysentery and diarrhoea. Several oligosaccharides comprising fructose, xylose, galactose, glucose and mannose occur naturally in banana (Debabandya et al., 2010) making it an excellent prebiotic for the selective growth of beneficial bacteria in the intestine. It aids in combating diarrhea and dysentery and promotes healing of intestinal lesions in ulcerative colitis. Roots of *Musa paradisiaca* are antihelmintic, flowers are astringent and fruits are mild laxative. It is also useful in celiac disease, constipation and peptic ulcer (Mallick et al., 2007). It has been found that bananas have curative properties both scientifically and traditionally. The banana flower is rich in phytochemicals like vitamins, flavonoids and proteins. The flower has been used to treat bronchitis, constipation and peptic ulcer. The extract has anti-oxidant property that prevents free radicals and control cell and tissue damage. Bhaskar et al., (2011) reported that glucose uptake in Ehrlich ascites tumor cells was stimulated by banana (Musa sp.) flower and pseudostem extracts. China et al., (2011) reported that, the banana flowers are a potential source of natural anti-oxidants.

Based on the main components of banana flower extract and its anti-oxidant properties, we hypothesized that the banana flower extract may have anticancer activities against cancer cell lines *in vitro*. However, there are no such reports.

Materials and Methods

Sample collection, authentication and preparation of extracts

The flowers of banana were collected from Bangalore, India on July–August 2011. The flower was separated and cut into small pieces and dried. The dried samples were ground into a fine powder using a dry grinder, and then kept in an air-tight container and stored in a freezer (-20°C) before extraction. 30 g of dried powder was used for serial extraction in a soxhlet apparatus using ethanol. The extracts were filtered and evaporated to dryness in a rotary evaporator. 1 mg/mL of the extract was prepared by dilution of the stock with sterile dimethyl sulfoxide (DMSO) (Duraipandiyan et al., 2006).

Chemicals

DMEM medium, fetal bovine serum (FBS), penicillin, streptomycin and MTT were procured from HIMEDIA (India). Caspase Apoptosis Assay Kit was purchased from G Biosciences (kit 786-205A), USA. All cell lines were bought from National Center for Cell Sciences (NCCS), Pune. All other chemicals and solvents used were of the highest purity grade available. Cell culture plastic ware was from Tarsons (India).

Cell lines and culture

HeLa and CHO cell lines were procured from National Center for Cell Sciences (NCCS), Pune. Cells were maintained in DMEM medium (HIMEDIA, REF – AT065A) supplemented with 10% fetal bovine serum (HIMEDIA, REF – RM112). Lymphocyte isolation was carried out from the blood of few healthy male and female individuals, about 20 years of age, who were free from infection for the past six months and had not been under any medication. HiSep medium (HIMEDIA, India) was used for the isolation. Lymphocytes were used as control cells to assess the cytotoxicity of the extracts on humans. The cells were incubated at 37°C with 95% air and 5% CO_2. All cells were maintained below passage 20 and used in experiments during the linear phase of growth.

MTT [3-(4, 5-dimethylthiazol-2yl)-2,5-diphenyl-tetrazolium bromide)] assay

HeLa and CHO cells growing exponentially were collected after trypsinization and plated in 96-well microtitre plates in 100 µL of culture medium and were allowed to adhere for 24 hours before treatment. Increasing concentrations of ethanol extract of banana flower dissolved in DMSO were added to different wells of the micro titer plates. Final concentration of DMSO in the culture medium was maintained at 0.4% (v/v) to avoid solvent toxicity. The cells were incubated for 24, 48 and 72 hours in the presence and absence of the extracts. Cytotoxicity was analyzed using MTT assay following the standard protocol (Mosmann et al., 1983). Cytotoxicity was expressed as the concentration of the extract inhibiting cell growth by 50%, relative to cells incubated in the presence of 0.4% DMSO. The absorbance was read at 540 nm using the ELISA plate reader. Each experiment was performed in triplicates.

The following formula was used to calculate the percent of inhibition:

$$\text{Inhibition (\%)} = (1\text{-}OD_s/OD) \times 100$$

Where, OD_s = Optical density of the sample; OD = Optical density of the control

Chromatographic separation of the bioactive compound (TLC)

Thin layer chromatography (TLC) was carried out using pre-coated TLC plates (Silica gel 60 F 254 Merck) to fractionate the bioactive components from the crude extracts. The silica gel coated sheet was activated at 110°C for 15 min. The extracts dissolved in ethanol (20 µL) were spotted at the bottom of silica gel coated sheet. Chromatogram was performed with the following solvent systems (a) toluene: ethyl acetate: formic acid (2.5:1:1 v/v); (b) chloroform : acetone (6:4 and 8:4 v/v); (c) hexane : acetone (6:4 and 8:2 v/v); (d) dichloromethane : acetone (6:4 and 8:2 v/v); (e) Toluene. The chromatograms were detected with the help of a UV transilluminator (254 and 366 nm) and the Rf value was calculated for each of the TLC separated fractions.

Detection of the bioactive fraction

For the detection of active compound separated in TLC, bioactivity guided fractionation was followed. From the chromatogram developed as described above, each band was scraped, mixed with methanol and centrifuged at 3,000 rpm for 15 min. Supernatant was collected in a pre-weighed vial and kept for evaporation. The partially purified fractions obtained from preparative TLC were tested for cytotoxicity against the HeLa cells, CHO cells and the lymphocytes by MTT assay as described earlier.

Fluorescence microscopic analysis by ethidium bromide/acridine orange (EB/AO) staining

HeLa and CHO cells growing exponentially were subcultured to 25 cm^2 culture flasks and were allowed to adhere for 24 hours before treatment. After this period, the cells were treated with bioactive fraction 2 from banana flower extract for 24 hours. After removal of the incubation medium cells were collected by trypsinization and treated with EB/AO stain. Stained cells were used to prepare slides and observed under a fluorescence microscope and were photographed (Cohen et al., 1993).

Caspase-9 activity assay

Caspase-9 activity was assessed using the caspase-9 Colorimetric Assay Kit (G Biosciences, kit 786-205A). The assay is based on spectophotometric detection of the chromophore *p*-nitro-aniline (*p*NA) after cleavage

from the labeled substrate LEHD-*p*NA. The free *p*NA can be quantified using a spectrophotometer or a microtiter plate reader at 405 nm. Comparison of the absorbance of *p*NA from an apoptotic sample with an uninduced control allows determination of the fold increase in caspase-9 activity.

Here, HeLa cells and CHO cells were exposed to the bioactive fraction 2 (20 µg/mL each) for 24 hours. Following treatment, the cells were subjected to caspase -9 activity assay following the manufacturer's instructions. Percentage increase was calculated using the following formula:

Percentage activity of caspase = $(OD_{control/sample} - OD_{blank})/OD_{blank} \times 100$

All experiments were performed in triplicate and repeated at least twice.

LDH cytotoxicity assay

LDH Assay is a colorimetric method of assaying cellular cytotoxicity. The assay quantitatively measures a stable cytosolic enzyme lactate dehydrogenase (LDH), which is released upon cell lysis. Cells treated with fraction 2 of banana flower for 24 hours were collected by tripsinization, centrifuged at 1,000 rpm for 10 min, and 10 µL of lysis buffer was added and plated in triplicates in a 96 well plate along with the controls, positive (1% Triton X-100) and negative (untreated). 50 µL of substrate was added to the wells and incubated in the dark for 20 min. After the incubation period, 50 µL of stop solution was added to all the wells to stop the reaction and readings were noted down at 490 nm. Percentage cytotoxicity was measured using the following formula:

Percentage cytotoxicity = $(OD_{treated} - OD_{negative\ control})/OD_{positive\ control} \times 100$

Cell cycle kinetics: Cells grown in 12-well plates (5.0 x 10^5 cells/mL) were treated with bioactive fraction 2 for 24 hours. Briefly, cell pellets were collected by tripsiniza -tion, washed twice with PBS and fixed overnight with 70% ethanol at 4°C. After incubation, cells were centrifuged again at 5,000 rpm for 10 min and washed twice with PBS. Cells were resuspended in 1 mL of PBS and in ribonuclease (100 µg/mL). Then cells were resuspended in staining solution [50 µg/mL propidium iodide, 30 units/mL RNase, 4 mM/L sodium citrate, and Triton X-100 (pH 7.8)] and incubated at 37°C for 15 min. After incubation in the dark, fluorescence-activeted cells were sorted in a FACScan flow cytometer (equipped with a 488 nm argon laser), and the data were analyzed on a MACS Quant analyser.

Phytochemical screening of the bioactive fraction

Preliminary qualitative phytochemical analysis of the banana flower crude extract and the bioactive fraction 2 were performed for the determination of the biochemical constituents with the help of standard protocols (Harborne, 1973). All the analyses were carried out using 1 mL of extracts. In the case of tests for carbohydrates, Fehling's and Benedict's tests were carried out. Tests for alkaloids (Wagner's and Mayer's tests), test for sterols (Salkowsky test), tests for the detection of phenolic compounds (test with neutral $FeCl_3$) and tannins, tests for proteins (Biuret test) and also tests for the detection of flavonoids (Shinoda test) and saponins, were performed.

HPLC analysis of the bioactive fraction

To further purify the active fractions, the TLC purified fraction of banana flowers were subjected to high performance liquid chromatography. The HPLC separation was performed using WATERS HPLC system with 2487 dual λ U-V detector. The sample and mobile phase were filtered through 0.22 µm PVDF filter before injecting to the column.

Spectroscopic analysis

The ESI mass spectra were recorded using a single quadrupole mass spectrometer (Hewlett Packard HP 1100 MSD series). Spectra were acquired over the mass range 50-1500 m/z.

Statistical analysis

All experiments were carried out in triplicates. The results were expressed as mean ± standard error values. Statistical significance was calculated using one- way analysis of variance (ANOVA) with the help of GraphPad Prism software. A value of $p<0.05$ was taken as statistically significant.

Results

When HeLa and CHO cells were treated with the ethanolic extracts of the banana flower, there was a dose and time dependent anti-proliferative effect observed in the case of HeLa cells. Percentage viability decreased as the period of exposure to the extract increased from 24 to 48 and 72 hours. At 5 µg/mL concentration of the extract, the percentage viability of 24 hours treated HeLa cells was 91% and it decreased to 50.0% by the end of 72 hours. As the concentration increased from 5 to 20 µg/mL, the percentage viability of HeLa cells further decreased to 38% (Figure 1). When CHO cells were treated with the ethanol extract of banana flower, we could see a decrease in percentage viability with an increase in concentration. At a concentration of 20 µg/mL, after 72 hours of treatment, the percentage viability was 60.0% as compared to 86% after 24 hours. It is evident that the effect was more profound on the HeLa cells than the CHO cells.

Chromatogram was developed with the solvent toluene for the extract. The chromatograms were detected with

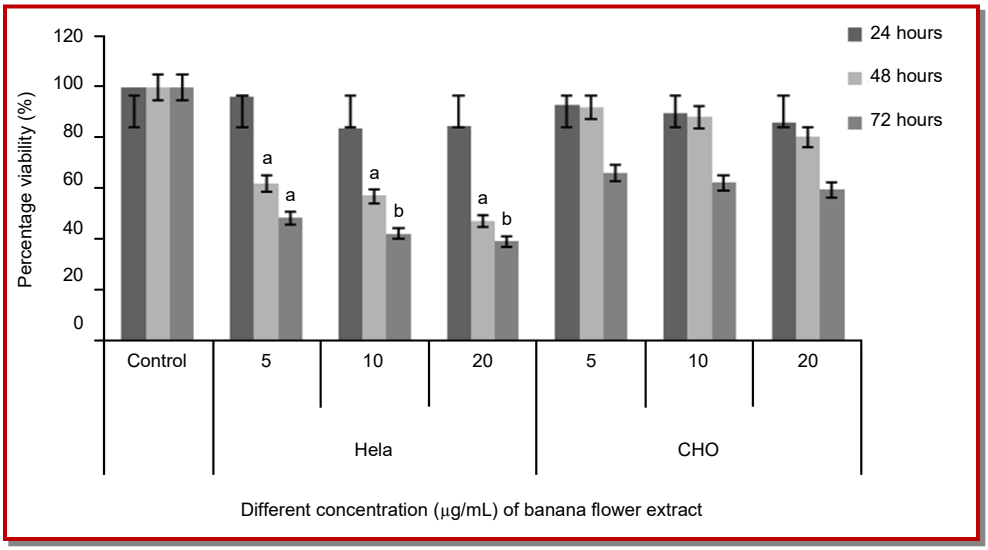

Figure 1: Effect of banana (*Musa paradisiaca*) flower ethanol extract on HeLa and CHO Cell Lines. [a]p<0.05 compared with control. [b]p<0.001 as compared to the control

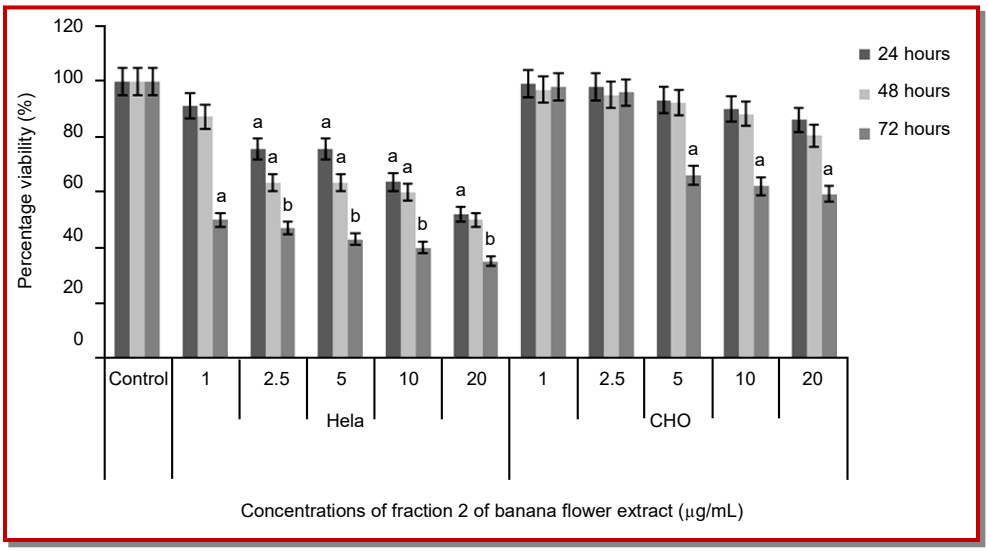

Figure 2: Effect of bioactive fraction 2 of banana flower extract on HeLa and CHO cells for 24, 48 and 72 hours. [a]p<0.05 compared with control. [b]p<0.001 as compared to the control

the help of a UV transilluminator (254 and 366 nm). Three major fractions were separated from the extract. The Rf values were 0.06, 0.47 and 0.72.

Fractions recovered from TLC were further tested for cytotoxicity by the MTT assay. The 2nd fraction of the ethanol extract of banana flower exhibited highest cytotoxic activity than the other fractions. Fraction 2 reduced the percentage viability of CHO cells to 60% and HeLa cells to 35% at 20 µg/mL concentration, after 72 hours of treatment (Figure 2). IC_{50} value was found to be 10 µg/mL for this bioactive fraction.

When the bioactive fraction 2 from the flower extract was checked for cytotoxicity on normal human peripheral lymphocytes, there was not much difference in the percentage viability at all the tested concentrations and at 20 µg/mL, the percentage viability was seen to be decreased to 92% at 72 hours, indicating that this fraction would be comparatively safer on humans (Figure 3).

By phytochemical screening, among the different tests performed, the samples (crude extract and the bioactive fraction 2) indicated the presence of phenols (results not shown). Based on the observation, the functional group of the active component was tentatively identified as a phenol.

Staining with EB/AO of the HeLa cells treated with the bioactive fraction from the banana flower extract, showed viable cells in the control flasks as green

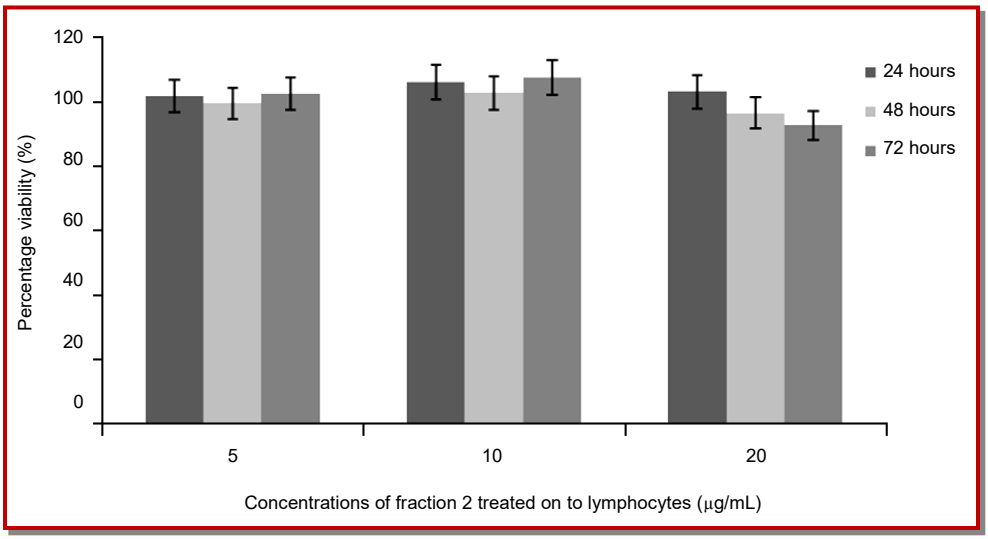

Figure 3: Effect of the bioactive fraction 2 on lymphocytes

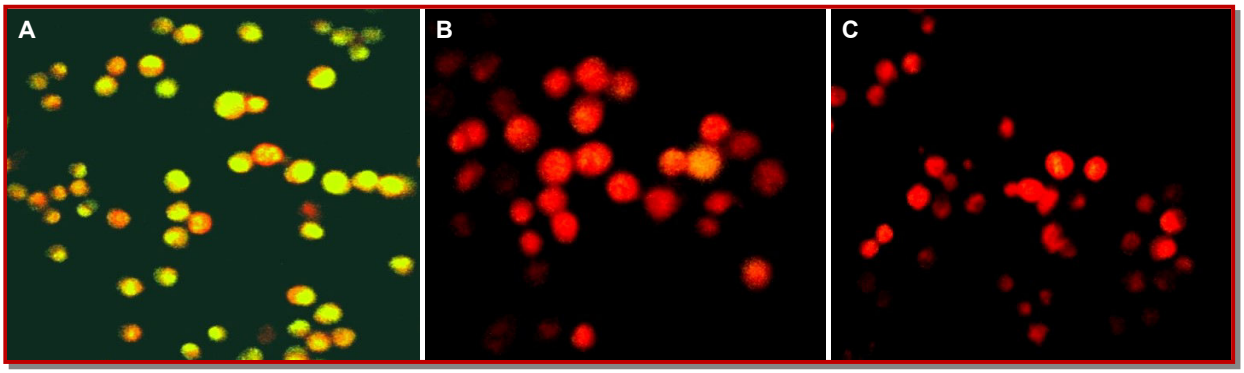

Figure 4: Fluorescence Microscopic photographs. (A) Control HeLa cells (B) HeLa treated with 10 µg/mL of bioactive fraction 2. (C) HeLa cells treated with 20 µg/mL of bioactive fraction 2. Arrows indicate the breaking up of the nuclei in apoptotic cells. The treated cells are bright orange in color and fewer in number as compared to the control HeLa cells which are greenish in color and more in numbers

(Figure 4A) with intact nuclei and the non-viable cells

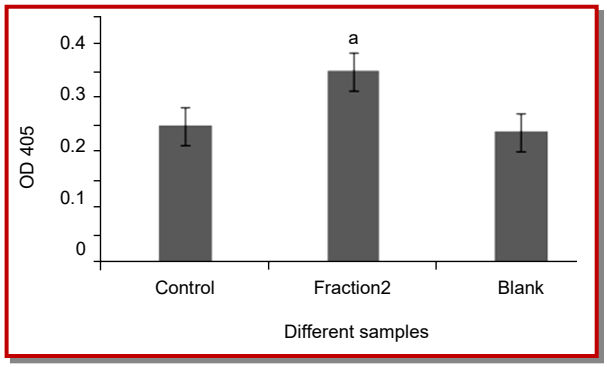

Figure 5: Comparision of Caspase level between Control and samples. [a]denotes 0.01 level of significance

in the treated flasks were bright orange (Figures 4B and 4C). Apoptosis was visible by the appearance of breaking up of the nuclei. The effect of the bioactive fraction 2 from banana flower extract on the activity of initiator caspase-9 is shown in Figure 5. The fraction was found to significantly increase the activity of caspase-9 in HeLa cells treated for 48 hours. There was a 2-fold increase of caspase activity in the treated HeLa cells as compared to the control HeLa, confirming the apoptotic induction of cell death in the cervical cancer cell line HeLa.

The cytotoxicity, as assessed by LDH release by the HeLa cells treated with 10 µg/mL of fraction 2 for 24 hours, was 32.8% (Figure 6) when compared with that of the positive control i.e., 1% Triton-X 100, which could induce all of the HeLa cell death through cytotoxic effects. This indicates that the bioactive fraction has released LDH when added to the HeLa cells, and LDH release assays are an appropriate and possibly preferable means of measuring cellular cytotoxic reactions.

The bioactive fraction 2 of banana flower demonstrated anticancer activity by inhibiting the growth cycle of an immortal cancer cell line HeLa. The addition of the bioactive fraction resulted in decreasing the total percentage of viable cells to 40.74% with the cell

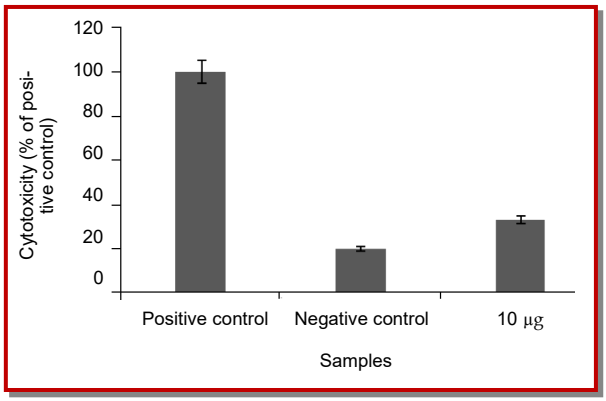

Figure 6: Cytotoxic effects of fraction 2 of banana flower extract determined by LDH leakage experiment using the cytotoxicity detection kit

ratio of 224.20 by ESI-MS analysis (Figure 8). This m/z value does not corresponding to that of any of the earlier reported anti-cancer bioactive compounds identified when checked in the anti-cancer database and hence appears to be a novel one.

Discussion

In this study we evaluated the anticancer potential of banana flower ethanol extract on the cervical cancer cell line HeLa. As it has exhibited very good cytotoxic and antiproliferative effects, we further purified it by TLC. Three fractions were separated and all were tested for antiproliferative properties on HeLa, CHO cells and normal human lymphocytes. Fraction 2 was showing

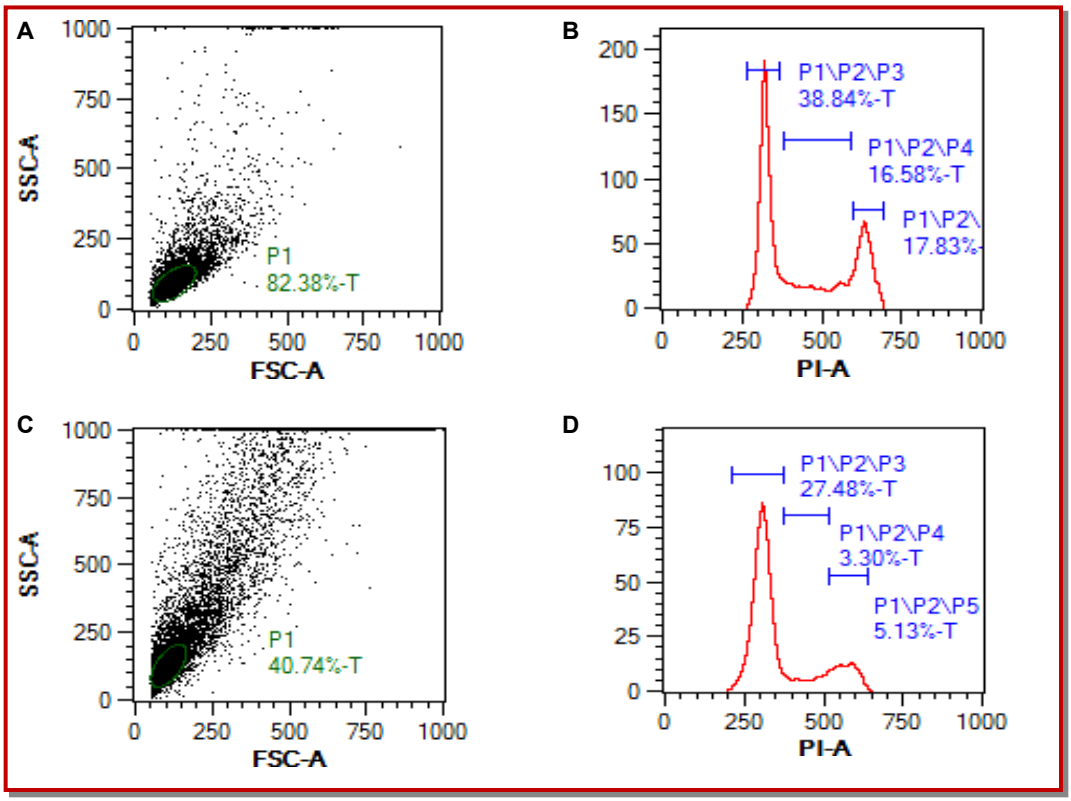

Figure 7: Cell cycle analysis of the HeLa cells. A and B: control HeLa cells. C and D: HeLa treated with bioactive fraction 2

population in the G_0/G_1 phase at 27.5% (Figure 7C and D), S phase at 3.3% and G_2/M phase with 5.1% when assessed after 24 hours of treatment. There were significantly fewer cells in the S and G2/M phases as compared to the controls (Figure 7A and B).

The bioactive fractions separated by TLC and identified by bio-assay guided fractionation were further characterized by LC-MS analysis. When the bioactive fraction 2 was subjected to HPLC, there were 3 peaks, one at RT 4.9, second larger peak at RT 6.9 and third at RT 10.1. The larger peak has shown a mass to charge

best results with an IC_{50} of <10 µg/mL concentration. This fraction was found to be a phenol by phytochemical screening. This bioactive fraction could induce apoptosis in the treated HeLa cells as evidenced by the 2-fold increase in caspase-9 activity assay as compared to the control Hela cells. The apop-tosis induction ability was more profound on HeLa rather than CHO cells. When the bioactive fraction 2 was subjected to HPLC, there were 3 peaks, one at RT 4.9, second larger peak at RT 6.9 and a third one at RT 10.1. The larger peak has shown a mass to charge ratio of 224.2. This molecular weight is not corresponding to

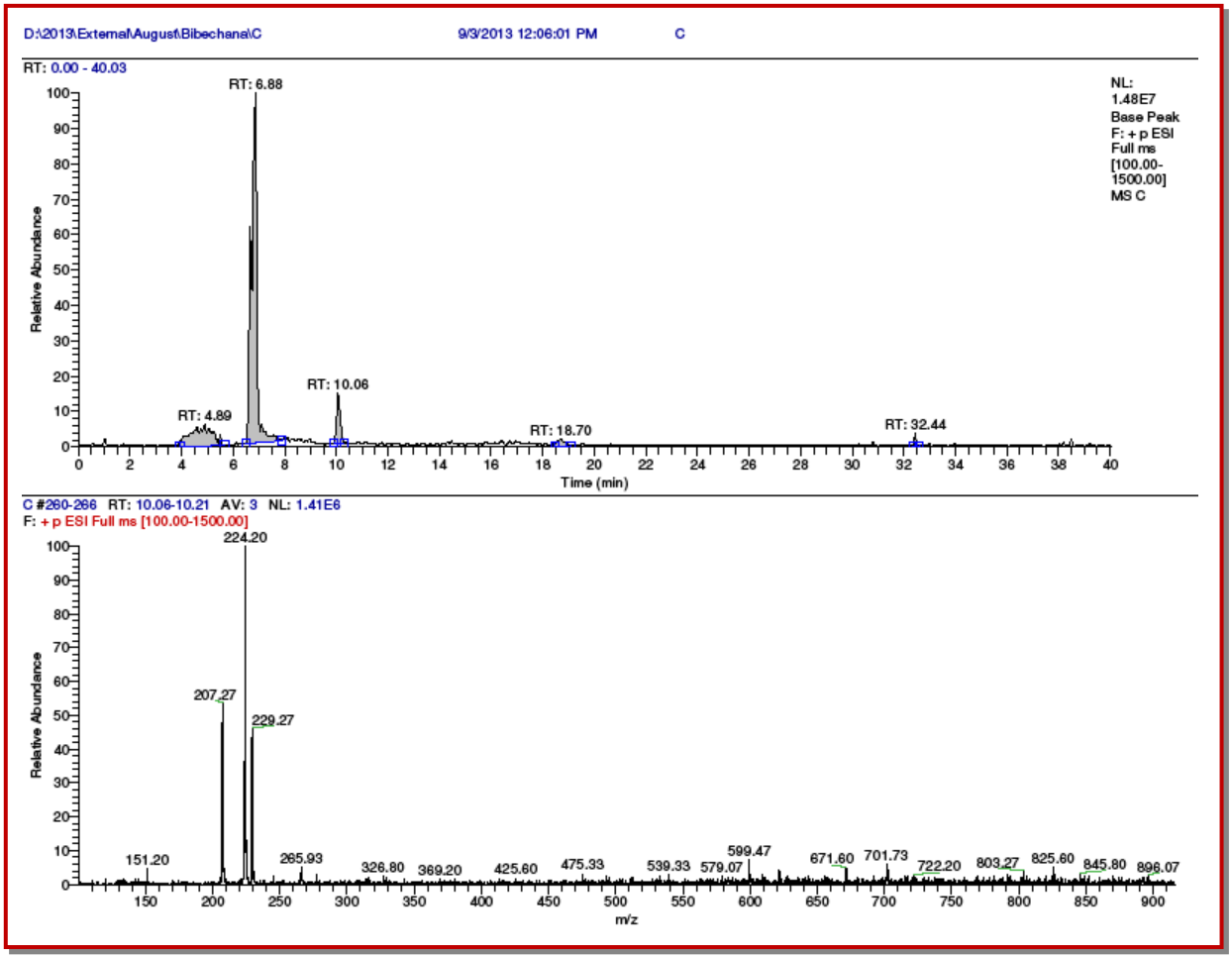

Figure 8: LC-MS analysis of TLC purified fraction 2 of banana flower extract
Upper panel: HPLC chromatogram of fraction 2 showing 3 peaks. Second peak was largest at RT 6.88 min.; Lower panel: LC-MS spectra of the second fraction, m/z 224.20

that of any other earlier reported bioactive compounds, so it could be a novel one with higher anticancer and anti-proliferative effects. Furthermore, this fraction was least toxic to human lymphocytes indicating that this can serve as a better and safer source for identifying a lead molecule in anti-cancer drug development. However, further characterization and animal studies are required to prove this. Till now there are several reports on the pharmacological properties of banana which include compounds that have anti-diabietic, anti-oxidant (dopamine) antimicrobial, and antiulcer (leucocyanidin) activities (Kanazawa and Sakakibara, 2000). Antifungal and antibiotic principles are found in the peel and pulp of fully ripe bananas. The antibiotic acts against Mycobacteria. A fungicide in the peel and pulp of green fruits is active against a fungus disease of tomato plants. Along with other fruits and vegetables, consumption of bananas is associated with a reduced risk of colorectal cancer (Deneo-Pellegrini et al., 1996), renal cell carcinoma (Rashidkhani et al., 2005) and breast cancer in women (Zhang et al., 2009). Banana stem extract from the Musaceae family had been suggested to be a useful agent in the treatment of patients with hyperoxaluric urolithiasis (Poonguzhali and Chegu, 1994), kidney stones and high blood pressure. Oral administration of chloroform extract of the *M. sapientum* flowers had been found to cause a significant reduction in blood glucose and glycosylated hemoglobin, increase in total hemoglobin and prevents decrease in body weight (Pari and Uma-Maheswari, 1999). Though there are some studies on the bioactivity of different parts of Musa species, the anti-cancer potential of banana flower extract has not been investigated so far.

This study shows that banana flower can serve as a very good natural source for the development of an anti -cancer lead molecule with least side effects.

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